Interaction of α_2-macroglobulin-bound thrombin with hirudin

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The human thrombin bound to α_2-macroglobulin (α_2 M) in a 1:1 stoichiometry is still able to interact with one of its specific inhibitors, hirudin. The dissociation constant of the complex hirudin–α_2M-bound thrombin is 1×10^{-7} M, whatever the mode of thrombin binding, covalent or non-covalent.

α_2-Macroglobulin *Thrombin* *Hirudin*

1. INTRODUCTION

Human α_2-macroglobulin (α_2M) is a plasma glycoprotein of M_r 725000 composed of two subunits, each containing two identical chains linked by disulfide bridges. α_2M forms enzymatically active complexes with nearly all endoproteases. The protease binding initiates a drastic conformational change of the α_2M molecule (reviews [1,2]). It has been proposed that the proteases are entrapped within the α_2M molecule as they lose the ability to react with large substrates or inhibitors [3], although the active site is not involved by complex formation. However, several investigations [4,5] have shown that the inaccessibility of the active center of the bound enzymes by large molecules is a relative rather than an absolute restrictive phenomenon. Several observations thus argue against a molecular trap hypothesis as a proven fact [6].

However, we failed to observe a reaction of α_2M-bound thrombin with antithrombin III [7]. Here, we investigate such an interaction using hirudin, an inhibitor of thrombin, with a lower molecular mass and an affinity constant for the free enzyme [8] greater than that of AT III [9].

2. EXPERIMENTAL

2.1. *Materials*

Human α_2M was prepared by Zn^{2+} affinity chromatography as in [10]. Thrombin was obtained as in [11]; the enzyme had a specific activity of 3200 units/mg. Hirudin was from Serva and had a specific activity of 1500 units/mg.

2.2. *Methods*

The enzymatic activity of thrombin was evaluated by the amidolytic activity of the enzyme, monitoring on a Cary 118 C at 25°C the hydrolysis rate of the chromogenic substrate H.D.Pip.Arg. pNA (Kabi Kiagnostica, Sweden).

The activity of the hirudin preparation was determined according to [8]. Thrombin was labelled by fluorescein isothiocyanate as in [7].

The α_2M–thrombin complexes were prepared by adding the protease at a 1×10^{-5} M α_2M preparation in 0.1 M NaCl, 0.05 M phosphate (pH 7.5). The reaction was sometimes performed in the presence of 0.1 M hydroxylamine [11]. The complexes were isolated by Sephacryl S 200 filtration in the same buffer.

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3. RESULTS

The specific and stoichiometric reaction of hirudin (M_r 10800) with human thrombin achieves inactivation of the clotting activity of the protease as well as of its amidolytic activity on synthetic substrates [8]. The association rate is so fast that, even in the nanomolar range, the rate association constant cannot be accurately determined. On the other hand, a 100-fold molar excess of α_2M is unable to dissociate the complex even after a 48 h incubation at 25°C. Indeed, no amidolytic activity corresponding to an eventually formed α_2M–thrombin complex can be detected.

As shown in fig.1, the rate of inhibition of the enzymatic activity of the α_2M-bound thrombin is very low compared to that of the free enzyme, equilibrium only being reached after several hours of incubation, even in the presence of a large excess of inhibitor. The inset of the figure is a plot of the thrombin activity as a function of hirudin concentration after equilibrium has been reached. From these data, a dissociation constant is calculated, $K_i = 1 \times 10^{-7}$ M. The value of this constant is not significantly different whatever the binding mode of thrombin to α_2M, covalent or non-covalent.

To exclude the possibility of the formation of an inactive thrombin–hirudin complex after the non-covalently bound thrombin has been displaced from its α_2M complex by hirudin, thrombin was first labelled by fluorescein isothiocyanate. Then the protease is reacted with α_2M and the complex incubated with a 100-fold molar excess of hirudin for 5 h and the reaction mixture chromatographed on Sephacryl S 200 (fig.2). As expected, labelled thrombin is eluted associated with the α_2M bulk using the covalent α_2M–thrombin complex, whereas only a few percent of the thrombin molecules are eluted as an inactive thrombin–hirudin conjugate, using the non-covalent α_2M–protease complex obtained in the presence of hydroxylamine. Then, the observed dissociation constant is really that of the ternary complex α_2M-bound thrombin–hirudin.

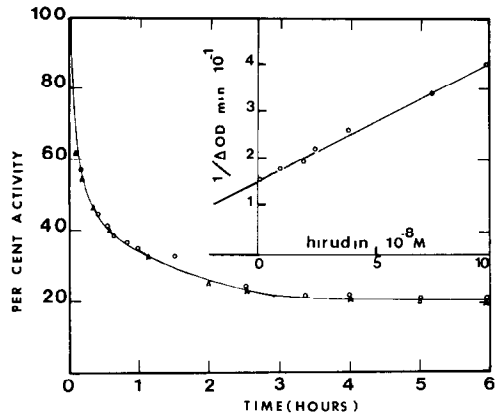

Fig.1. Time dependency of the inhibition of α_2M-bound thrombin by hirudin at pH 7.5 and 25°C. Hirudin at a final concentration of 5×10^{-7} M is incubated with the α_2M–thrombin complex at 1×10^{-8} M. The activity of thrombin is tested at the indicated times. (○---○) α_2M covalently bound thrombin, (△---△) α_2M non-covalently bound thrombin. The inset is a Dixon plot of the residual activity of the α_2M–thrombin complex for different concentrations of hirudin after equilibrium has been reached (5 h at 25°C).

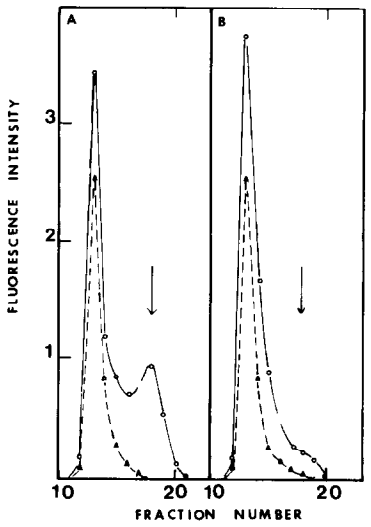

Fig.2. Elution profiles of α_2M-bound thrombin–hirudin complexes. The α_2M–thrombin complexes are incubated at 25°C for 10 h with a 100-fold molar excess of hirudin and chromatographed on Sephacryl S-200. The arrows correspond to the expected elution peak of the hirudin–thrombin complex. (A) and (B) are the non-covalent and covalent α_2M–thrombin complexes, respectively. The elution profiles are performed using the fluorescence of fluorescein-labelled thrombin (○—○); the residual activity of the bound protease is also tested (△---△).

4. DISCUSSION

The interaction of hirudin with the α_2M thrombin complex is very much slower than with the free enzyme. This difference in reaction rate probably did not allow authors in [12] to observe this interaction. Furthermore, a 5-fold molar excess of hirudin is necessary to detect a significant inhibition. The factors causing this drastic reduction rate may reflect the limit of accessibilty of α_2M-bound proteases. Indeed, AT III, with a molecular mass nearly 6-fold greater, does not react with the conjugated thrombin even in the presence of heparin [11]. But other factors may be involved, for instance, the value of the affinity constant of the inhibitor for the protease: that of hirudin being considerably greater than that of AT III for free thrombin [8,9]. Furthermore, a conformational modification of the surrounding of the active center of the protease upon α_2M binding cannot be excluded.

It may also be pointed out that, whatever the binding mode of thrombin to α_2M, covalent or not, the K_i for the hirudin–thrombin interaction is identical: the accessibility of the bound protease and/or its affinity constant for hirudin are not modified, supporting the idea that the absence of covalent bonds does not modify the flexibility of the α_2M–thrombin complex and, in any case, provides additional support for identical α_2M protease binding sites [7].

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